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CHAPTER 5

THE C_{ss}RS TWO-COMPONENT REGULATORY SYSTEM CONTROLS A GENERAL SECRETION STRESS RESPONSE IN *BACILLUS SUBTILIS* UNDER SECRETION STRESS CONDITIONS

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SUMMARY

Bacillus species are valuable producers of industrial enzymes and biopharmaceuticals, because they can secrete large quantities of high-quality proteins directly into the growth medium. This requires the concerted action of quality control factors, such as folding catalysts and 'cleaning proteases'. The expression of two important cleaning proteases, HtrA and HtrB, of *Bacillus subtilis* is controlled by the CssRS two-component regulatory system. The induced CssRS-dependent expression of *htrA* and *htrB* has been defined as a protein secretion stress response, because it can be triggered by high-level production of secreted α -amylases. It was not known whether translocation of these α -amylases across the membrane is required to trigger a secretion stress response or whether other secretory proteins can also activate this response. These studies show for the first time that the CssRS-dependent response is a general secretion stress response which can be triggered by both homologous and heterologous secretory proteins. As demonstrated by high-level production of a non-translocated variant of the α -amylase, AmyQ, membrane translocation of secretory proteins is required to elicit this general protein secretion stress response. Studies with two other secretory reporter proteins, lipase A of *B. subtilis* and human interleukin-3, show that the intensity of the protein secretion stress response only partly reflects the production levels of the respective proteins. Importantly, degradation of human interleukin-3 by extracellular proteases has a major impact on the production level, but only a minor effect on the intensity of the secretion stress response.

INTRODUCTION

Bacillus subtilis is a Gram-positive, non-pathogenic organism which is widely used for the production of industrially important enzymes. A major advantage of this organism is its ability to secrete proteins directly into the growth medium, which facilitates the subsequent product purification. In general, the quality of proteins exported into the growth medium is high, which can be attributed to the quality control systems of *B. subtilis*. These systems consist of foldases and proteases that are involved in the correct folding of proteins and/or the removal of incompletely synthesised, damaged or misfolded proteins in the different compartments of the cell (Jensen *et al.*, 2000; Kruger *et al.*, 2000; Hyyryläinen *et al.*, 2001; Chapter 2, Westers *et al.*, 2004b). By studying the quality control systems of *B. subtilis* in more detail, various key players in the complex Sec-dependent protein secretion machinery have been identified (Tjalsma *et al.*, 2000; Sarvas *et al.*, 2004; Chapter 2, Westers *et al.*, 2004b).

Although the secretion of homologous proteins by *B. subtilis* is generally very efficient, various yield-limiting bottlenecks for efficient secretion of proteins from especially Gram-negative eubacterial or eukaryotic origin were identified (Bolhuis *et al.*, 1999a). Firstly, heterologous proteins may form insoluble aggregates in the cytoplasm (Wu *et al.*, 1998). Secondly, they may be poorly targeted to the membrane or rejected by the preprotein translocation system in the membrane (Tjalsma *et al.*, 2000). Thirdly, after the translocation process, proteins may be degraded by membrane-bound, cell wall-associated or secreted proteases of *B. subtilis*. This degradation may relate either to slow or incorrect posttranslocational folding or the presence of exposed protease-recognition sequences in the folded protein (Simonen and Palva, 1993; Sarvas *et al.*, 2004).

The Sec machinery seems to be responsible for the export of most proteins from the cytoplasm of *B. subtilis* (Tjalsma *et al.*, 2004). As documented for the *Escherichia coli* Sec translocase, this machinery can only handle proteins in an unfolded state (de Keyzer *et al.*, 2003). As unfolded proteins are particularly susceptible to proteolysis, the translocated proteins that emerge from the Sec translocation channel must fold efficiently into their native conformation at the membrane-cell wall interface (Sarvas *et al.*, 2004). Thereafter, they can pass the cell wall in order to be released into the growth medium. During these post-translocational stages in protein secretion, prominent roles are played by the folding catalyst PrsA (Kontinen *et al.*, 1991), various thiol-disulphide oxidoreductases (Bolhuis *et al.*, 1999b), and negatively charged cell wall

polymers (Stephenson and Harwood, 1998; Hyyryläinen *et al.*, 2000). The PrsA protein, which is anchored to the membrane via a lipid modification, has been shown to be particularly important for the folding and stability of many exported proteins at the membrane-cell wall interface (Jacobs *et al.*, 1993; Leskelä *et al.*, 1999; Hyyryläinen *et al.*, 2000 and 2001; Vitikainen *et al.*, 2001). Despite the presence of effective folding catalysts at this subcellular location, protein misfolding and/or aggregation cannot always be prevented by the cell. These misfolded or aggregated proteins are removed by membrane and cell wall-associated 'cleaning proteases' (Meens *et al.*, 1997; Bolhuis *et al.*, 1999a; Hyyryläinen *et al.*, 2001; Stephenson *et al.*, 2002), such as the membrane-associated HtrA and HtrB proteases of *B. subtilis* (Antelmann *et al.*, 2003; D. Noone and K. Devine, personal communication). Notably, HtrA (**H**igh-**t**emperature **r**equirement **A**) has a dual localisation, because it can be detected in the membrane-associated cellular fraction as well as the growth medium (Antelmann *et al.*, 2003). The physiological relevance of HtrA secretion into the growth medium remains to be shown.

The expression of the *htrA* and *htrB* genes is controlled by the two-component system CssRS (**C**ontrol **s**ecretion **s**tress **R**egulator and **S**ensor) (Hyyryläinen *et al.*, 2001). Consequently, CssRS is a key determinant in the regulation of misfolded protein degradation at the membrane cell-wall interface, as clearly illustrated by high-level production of the α -amylase AmyQ of *Bacillus amyloliquefaciens* in a *prsA3-cssS* double-mutant strain (Hyyryläinen *et al.*, 2001). High-level production of this α -amylase, or the related α -amylase AmyL from *Bacillus licheniformis*, activates the transcription of *htrA*, *htrB*, and the *cssRS* operon using a relay of phosphorylation-dephosphorylation in the CssRS two-component system (Darmon *et al.*, 2002). Notably, induced high-level production of AmyQ in *prsA3-cssS* or *prsA3-cssR* double-mutant strains resulted in severe growth retardation and subsequent cell lysis, a phenomenon that was not observed upon high-level AmyQ production in the respective *prsA3*, *cssS* or *cssR* single mutant strains (note that the *prsA3* mutation results in a 10-fold reduction in the cellular concentration of the essential PrsA protein (Hyyryläinen *et al.*, 2001)). These findings showed that the stress imposed on the cell under conditions of highlevel AmyQ production is highly detrimental if an adequate CssRS-mediated response involving the induction of the HtrA and HtrB proteases is precluded. The stimuli that trigger the CssRS-mediated *htrA* and *htrB* expression at elevated levels have, collectively, been termed 'secretion stress'. Notably, a secretion stress response is not only provoked by the high-level production of α -amylases, but also by mutation of *htrA* or *htrB*, or by the exposure of *B. subtilis* to heat. From the currently available data, it seems most likely that unfolded proteins represent, directly or indirectly, the

stimuli for the *Bacillus* secretion stress response (Noone *et al.*, 2000 and 2001; Darmon *et al.*, 2002; Antelmann *et al.*, 2003; Hyyryläinen *et al.*, 2005).

Thus far, the only secretory proteins that have been documented to trigger a secretion stress response on high-level production have been the α -amylases AmyQ and AmyL (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). It remained unclear, however, whether a secretion stress response was exclusively elicited by translocated α -amylases, or also by α -amylase precursors before their translocation across the membrane. Furthermore, it was not clear whether α -amylases are the only secretory proteins that trigger a secretion stress response that results in the induction of *htrA* and *htrB*, or whether this would also be the case for other secretory proteins produced at high levels. The present studies aimed to answer these questions. Here we present the novel observations that a non-translocated α -amylase precursor does not trigger a secretion stress response, and that the C_{ss}RS-dependent response is a general secretion stress response.

EXPERIMENTAL PROCEDURES

Plasmids, bacterial strains, and media

Table 1 lists the plasmids and bacterial strains used. Luria Bertani medium contained Bacto tryptone (1 %), Bacto yeast extract (0.5 %), and NaCl (0.5 %). The medium that was used for overexpression of lipase (Lesuisse *et al.*, 1993), in this work referred to as 1x MXR (medium extra rich), contained Bacto yeast extract (2.4 %), casein hydrolysate (1.2 %), arabic gum (0.4 %), glycerol (0.4 %), 0.17 M KH₂PO₄, and 0.72 M K₂HPO₄. The 1x MSR (medium super rich) used for hIL-3 production contained Bacto yeast extract (2.5 %), Bacto tryptone (1.5 %), K₂HPO₄ (0.3 %), xylose (1.0 %), and glucose (0.1 %). Trace elements were added from a 1000x stock solution (2 M MgCl₂, 0.7 M CaCl₂, 50 mM MnCl₂, 5 mM FeCl₃, 1 mM ZnCl₂, and 2 mM thiamine). Antibiotics were used in the following concentrations: chloramphenicol (Cm), 5 μ g/ml; erythromycin (Em), 2 μ g/ml; kanamycin (Km), 30 μ g/ml; and spectinomycin (Sp), 100 μ g/ml. The presence of the *htrA*::pMutin2 or *htrB*::pMutin4 mutations was checked by plating on Luria Bertani agar supplemented with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 160 μ g/ml) and erythromycin. Transformants containing these mutations were blue and Em^r.

Strain construction

B. subtilis was transformed as described by Kunst and Rapoport (1995). The *B. subtilis* 168 derivatives, BV2002 (*htrA*::pMutin2 *cssS*::Sp) and BV2015 (*htrB*::pMutin4 *cssS*::Sp), were constructed by transformation of *B. subtilis* BV2003 (*htrA*::pMutin2) and BFA3041 (*htrB*::pMutin4), respectively, with chromosomal DNA of *B. subtilis* BV2001 (*cssS*::Sp) and selection for spectinomycin resistance. The *B. subtilis* strains LH800A (WB800 *htrA*::pMutin2) and LH800B (WB800 *htrB*::pMutin4) were constructed by transformation of *B. subtilis* WB800 with chromosomal DNA of, respectively, *B. subtilis* BV2003 (*htrA*::pMutin2) or *B. subtilis* BFA3041 (*htrB*::pMutin4). Correct transformants were blue and Em^r. The strains obtained were transformed with chromosomal DNA of *B. subtilis* BV2001 (*cssS*::Sp) and selected for spectinomycin resistance to obtain the *B.*

subtilis strains LH800AS (WB800 *htrA*::pMutin2 *cssS*::Sp) and LH800BS (WB800 *htrB*::pMutin4 *cssS*::Sp).

Table 1. Plasmids and strains

	Relevant properties ^a	References
Plasmids		
pLip2031	pUB110 derivative; carries the <i>B. subtilis</i> <i>lipA</i> gene under the control of the HpaII promoter; Km ^r	Dartois <i>et al.</i> , 1994
pKTH10L	pUB110 derivative containing the <i>amyQ</i> gene of <i>B. amyloliquefaciens</i> ; Km ^r	Hyryläinen <i>et al.</i> , 2001
pKTHM101	pUB110 derivative containing the <i>amyQ</i> gene of <i>B. amyloliquefaciens</i> , encoding AmyQ with an artificial leucine-rich signal peptide; Km ^r	Zanen <i>et al.</i> , 2005
pKTHM102	pUB110 derivative containing the <i>amyQ</i> gene of <i>B. amyloliquefaciens</i> , encoding AmyQ with an artificial alanine-rich signal peptide; Km ^r	Zanen <i>et al.</i> , 2005
pP43LatIL3	pMA5 derivative containing the <i>hIL-3</i> gene with the <i>amyL</i> signal sequence, downstream of the HpaII and P43 promoters; Km ^r	Chapter 3, Westers <i>et al.</i> , 2006b
Strains of <i>B. subtilis</i>		
168	<i>trpC2</i>	Kunst <i>et al.</i> , 1997
168 <i>cssS</i>	Also known as BV2001; <i>trpC2</i> ; <i>cssS</i> ::Sp; Sp ^r	Hyryläinen <i>et al.</i> , 2001
168 <i>htrA-lacZ</i>	Also known as BV2003; <i>trpC2</i> ; <i>htrA</i> ::pMutin2; Em ^r	Hyryläinen <i>et al.</i> , 2001
168 <i>htrB-lacZ</i>	Also known as BFA3041; <i>trpC2</i> ; <i>htrB</i> ::pMutin4; Em ^r	Darmon <i>et al.</i> , 2002
168 <i>htrA-lacZ cssS</i>	Also known as BV2002; <i>trpC2</i> ; <i>htrA</i> ::pMutin2; <i>cssS</i> ::Sp; Em ^r ; Sp ^r	Hyryläinen <i>et al.</i> , 2001
168 <i>htrB-lacZ cssS</i>	Also known as BV2015; <i>trpC2</i> ; <i>htrB</i> ::pMutin4; <i>cssS</i> ::Sp; Em ^r ; Sp ^r	Darmon <i>et al.</i> , 2002
WB800	<i>nprE</i> ; <i>nprB</i> ; <i>aprE</i> ; <i>epr</i> ; <i>mpr</i> ; <i>bpf</i> ; <i>vpr</i> ; <i>wprA</i> ; Cm ^r ; Hyg ^r	Wu <i>et al.</i> , 2002
WB800 <i>htrA-lacZ</i>	Also referred to as LH800A; <i>nprE</i> ; <i>nprB</i> ; <i>aprE</i> ; <i>epr</i> ; <i>mpr</i> ; <i>bpf</i> ; <i>vpr</i> ; <i>wprA</i> ; <i>htrA</i> ::pMutin2; Cm ^r ; Hyg ^r ; Em ^r	This work
WB800 <i>htrB-lacZ</i>	Also referred to as LH800B; <i>nprE</i> ; <i>nprB</i> ; <i>aprE</i> ; <i>epr</i> ; <i>mpr</i> ; <i>bpf</i> ; <i>vpr</i> ; <i>wprA</i> ; <i>htrB</i> ::pMutin4; Cm ^r ; Hyg ^r ; Em ^r	This work
WB800 <i>htrA-lacZ cssS</i>	Also referred to as LH800AS; <i>nprE</i> ; <i>nprB</i> ; <i>aprE</i> ; <i>epr</i> ; <i>mpr</i> ; <i>bpf</i> ; <i>vpr</i> ; <i>wprA</i> ; <i>htrA</i> ::pMutin2; <i>cssS</i> ::Sp; Cm ^r ; Hyg ^r ; Em ^r ; Sp ^r	This work
WB800 <i>htrB-lacZ cssS</i>	Also referred to as LH800BS; <i>nprE</i> ; <i>nprB</i> ; <i>aprE</i> ; <i>epr</i> ; <i>mpr</i> ; <i>bpf</i> ; <i>vpr</i> ; <i>wprA</i> ; <i>htrB</i> ::pMutin4; <i>cssS</i> ::Sp; Cm ^r ; Hyg ^r ; Em ^r ; Sp ^r	This work

^aKm^r, kanamycin resistance marker; Em^r, erythromycin resistance marker; Cm^r, chloramphenicol resistance marker; Sp^r, spectinomycin resistance marker.

SDS-PAGE, Western blotting, and immunodetection

To detect overproduced and secreted LipA, hIL-3, or AmyQ, *B. subtilis* cells were separated from the growth medium by centrifugation (2 min at 5000 g, followed by 2 min at 13000 g at room temperature). Samples for SDS-PAGE were prepared as described previously (Van Dijk *et al.*, 1991). After separation by SDS-PAGE, proteins were stained with Coomassie Brilliant Blue (Neuhoff *et al.*, 1988) or transferred to a Protran® nitrocellulose transfer membrane (Schleicher and Schuell, the Netherlands) as described by Kyhse-Andersen (1984). AmyQ was detected with specific

antibodies and anti-rabbit IgG conjugates (Biosource International, USA). The alkaline phosphatase conjugate was detected using a standard NBT-BCIP reaction (Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate; Duchefa Biochemistry, the Netherlands) (Sambrook *et al.*, 1989). Densitometric analyses of stained gels were performed using the GENETOOLS software of the Chemigenius2 XE (Syngene, UK) image acquisition system.

Assays of enzyme activity

For strains containing a transcriptional *lacZ* fusion, the β -galactosidase assay and the calculation of β -galactosidase units (Miller units: nmol/min/A₆₀₀) were performed with the protocol used by Hyryläinen *et al.* (2001). Overnight cultures were diluted in fresh medium and samples were taken at different intervals for absorbance readings at 600 nm and β -galactosidase activity determinations. To assay β -galactosidase activity, a semi-automated method was developed, using a MultiPROBE®IIex Robotic Liquid Handling System (Perkin Elmer, USA). From the samples, treated with lysis buffer as described by Hyryläinen *et al.* (2001), an aliquot of 25 μ l was transferred to flat-bottom 96-wells plate (Greiner Bio-One, the Netherlands) in triplicate. The reaction was started by the addition of 100 μ l Z-buffer with dithiothreitol (1 mM final concentration) and o-nitrophenol galactoside (1 mg/ml final concentration) at 28 °C. After 15, 30 and 60 min the reaction was stopped by adding 62.5 μ l 1 M Na₂CO₃. β -Galactosidase activity was determined by measuring the increase in A₄₂₀. The measurements stopped after 60 min were used for further analyses, unless the A₄₂₀ was too high and therefore not reliable. Experiments were performed at least in duplicate starting with independently obtained transformants. In all experiments, the relevant controls were performed in parallel. The transition point between the exponential and post-exponential growth phases ($t = 0$) of every culture was determined individually, after which the corresponding LacZ activities were plotted in relation to $t = 0$. Although some differences were observed in the absolute β -galactosidase activities, the ratios between these activities in the various strains tested were largely constant. As a positive control, the pKTH10L plasmid directing AmyQ expression was introduced in all indicator strains, and AmyQ was shown to induce a C_{ss}RS-dependent secretion stress response. Points in the growth curves with an A₆₀₀ < 0.1 were omitted from the final datasets.

To determine lipase activity, the colorimetric assay as described by Lesuisse *et al.* (1993) was applied with some modifications. In short, a semi-automated analysis was performed, using a MultiPROBE®IIex Robotic Liquid Handling System (Perkin Elmer), in which 180 μ l of reaction buffer (0.1 M potassium phosphate buffer, pH 8.0, 0.1 % Arabic gum, 0.36 % Triton X-100) was supplemented with 10 μ l of the substrate 4-nitrophenyl caprylate (10 mM in methanol). The reaction was started by the addition of 10 μ l culture supernatant. Lipase activity was determined by measuring the increase in A₄₀₅ per min of incubation at room temperature, per A₆₀₀ of the culture at the time of sampling. Experiments were performed with growth medium fractions of at least four different pLip2031 transformants per tested strain. The lipase activity in each growth medium fraction was determined in triplicate (Westers *et al.*, 2005).

RESULTS

Non-translocated pre-AmyQ does not provoke a secretion stress response

Previous studies with AmyQ and AmyL as model proteins have shown that high-level production of these proteins in *B. subtilis* 168 provokes a C_{ss}RS-dependent secretion stress response (Hyryläinen *et al.*, 2001; Darmon *et al.*,

2002). To investigate whether this secretion stress response is triggered by translocated or non-translocated α -amylase, the authentic pre-AmyQ and two derivatives of this preprotein with mutated signal peptides were used. The two mutated signal peptides of AmyQ that were used contain either a stretch of leucines or a stretch of alanines, resulting in more hydrophobic (AmyQ-Leu) or less hydrophobic (AmyQ-Ala) signal peptides, respectively (Zanen *et al.*, 2005). As shown by Western blotting, authentic AmyQ and AmyQ-Leu were secreted into the growth medium, whereas no mature AmyQ-Ala was secreted (Fig. 1A). In fact, all AmyQ-Ala detectable in the cells was present in the precursor form and localised in the cytoplasm (Zanen *et al.*, 2005). Notably, compared with the authentic AmyQ, lower amounts of AmyQ-Leu and higher amounts of AmyQ-Ala were present in the cells. Cells from *B. subtilis* 168 *htrA-lacZ*, or 168 *htrB-lacZ* strains overexpressing AmyQ-Leu or AmyQ-Ala were used to determine whether these proteins induce a secretion stress response like the authentic AmyQ. Furthermore, the effects of AmyQ-Leu or AmyQ-Ala production were tested in *cssS* mutant control strains to verify the C_{ss}S dependence of *htrA-lacZ* or *htrB-lacZ* expression. It should be noted that, because of the way in which the transcriptional *htrA-lacZ* or the *htrB-lacZ* reporter gene fusions have been constructed, either the *htrA* gene or the *htrB* gene is disrupted in the respective indicator strains (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). This renders these indicator strains more responsive to secretion stress, as *htrA* and *htrB* expression is negatively autoregulated and reciprocally cross-regulated (Noone *et al.*, 2001). Consequently, the *htrA-lacZ* or *htrB-lacZ* indicator strains are perfectly suited for the detection of relatively mild secretion stress stimuli.

Whereas the production of (pre)AmyQ with the authentic signal peptide triggered a secretion stress response, represented by a large increase in the *htrB-lacZ* transcription (Fig. 1B, ■), the production of AmyQ-Ala did not provoke such a response (Fig. 1B, ◆). In fact, the level of *htrB-lacZ* transcription in cells producing AmyQ-Ala was comparable to the level observed in *htrB-lacZ* cells which do not produce AmyQ (data not shown). Production of AmyQ-Leu did trigger a secretion stress response (Fig. 1B, ▲), although the intensity of this response was lower than that provoked by high-level production of wild-type AmyQ. Importantly, the AmyQ-Leu-induced secretion stress response was completely C_{ss}RS-dependent (not shown), like the secretion stress response provoked by wild-type AmyQ (Fig. 1B, □). Fig. 1B documents only the results obtained with the *htrB-lacZ* gene fusion, but very similar results were obtained with the *htrA-lacZ* reporter gene fusion, which is consistent with the fact that AmyQ production results in the increased transcription of both *htrA* and *htrB* (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). Taken together, these

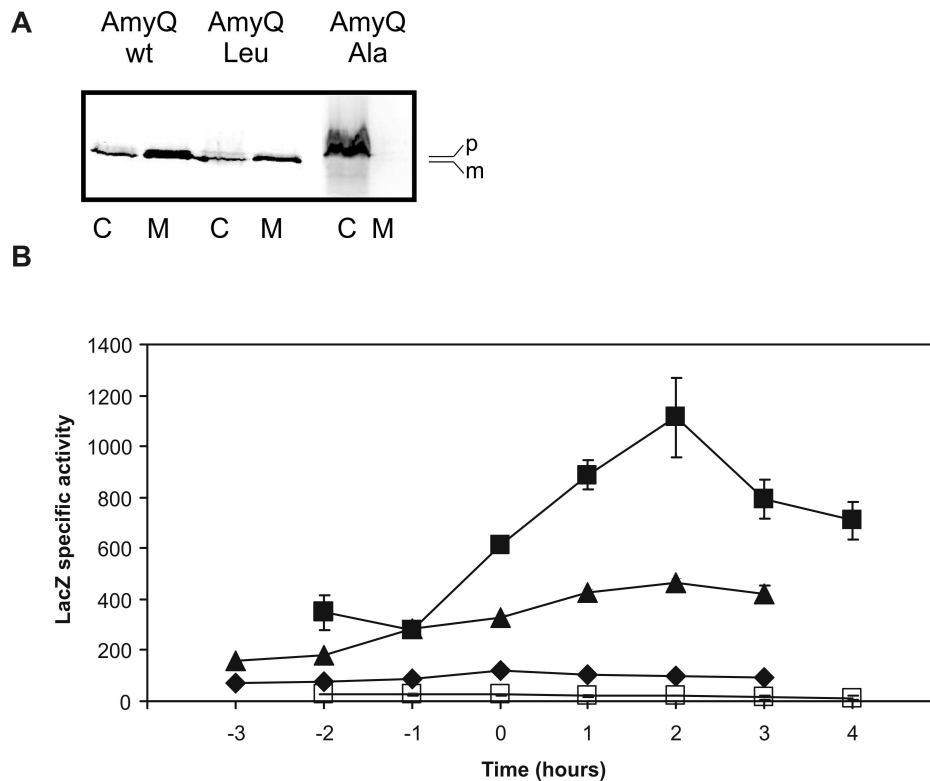


Figure 1. AmyQ-induced secretion stress response

(A) The concentrations of overproduced (pre)AmyQ were analysed by western blotting, using cellular (C) and/or growth medium (M) fractions of *B. subtilis* 168 pKTH10L (encodes wild-type AmyQ), *B. subtilis* 168 pKTHM101 (encodes AmyQ-Leu), and *B. subtilis* 168 pKTHM102 (encodes AmyQ-Ala). AmyQ was visualised with specific antibodies. The accumulation of high amounts of preAmyQ-Ala in the cells and complete absence of mature AmyQ-Ala in the medium fraction was verified in three independent biological replicates, one of which is shown here. p, precursor; m, mature.

(B) To compare the induction of secretion stress responses in *B. subtilis* 168 overexpressing wild-type AmyQ, AmyQ-Leu or AmyQ-Ala, a transcriptional *htrB-lacZ* fusion was used. Time courses of *htrB-lacZ* expression were determined by analysing β -galactosidase (LacZ) activity (indicated in nmol/min/A₆₀₀) in cells grown in Luria-Bertani medium at 37 °C. Samples were withdrawn at the times indicated; zero time is defined as the transition point between exponential and post-exponential growth. The strains used for the analyses were: *B. subtilis* 168 *htrB-lacZ* pKTH10L (produces wild-type AmyQ; ■); *B. subtilis* 168 *htrB-lacZ* *cssS* pKTH10L (produces wild-type AmyQ; □); *B. subtilis* 168 *htrB-lacZ* pKTHM101 (produces AmyQ-Leu; ▲); *B. subtilis* 168 *htrB-lacZ* pKTHM102 (produces AmyQ-Ala; ◆).

findings show that the non-translocated pre-AmyQ-Ala does not trigger a secretion stress response, whereas translocated AmyQ does elicit a secretion stress response. The intensity of the secretion stress response provoked by translocated AmyQ seems to correlate with the production level of this protein.

Deletion of multiple genes for extracellular proteases does not trigger a secretion stress response

Heterologous secretory proteins often need to be protected against degradation by the proteases that *B. subtilis* secretes into the growth medium in order to facilitate their high-level production. This can be achieved through the use of the protease-deficient strain WB800, which lacks eight important extracellular proteases (AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr, and WprA) (Wu and Wong, 2002). It should be noted that deletion of the wall-bound WprA, which has a processing product with proteolytic activity, generally known as CWBP52, will lead to a reduced protease activity in the cell wall of the WB800 strain (Margot and Karamata, 1996; Antelmann *et al.*, 2002). To investigate the influence of these eight extracellular proteases on the expression of *htrA* and *htrB*, the *htrA-lacZ* and *htrB-lacZ* transcriptional fusions were introduced into *B. subtilis* WB800. Interestingly, the *htrA-lacZ* and *htrB-lacZ* expression levels in *B. subtilis* WB800 and the parental strain 168 were very similar (Fig. 2), showing that the deletion of these proteases in *B. subtilis* WB800 on its own does not cause an obvious secretion stress response. Notably, in both strains, the basal level of *htrA-lacZ* expression was higher than that of *htrB-lacZ*. Moreover, the expression of the *htrB-lacZ* reporter gene fusion has previously been shown to be more sensitive to secretion stress than the *htrA-lacZ* reporter gene fusion (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). Therefore only the *htrB-lacZ* fusion was used as the preferred reporter of secretion stress in the further experiments of this study.

High-level lipase A (LipA) production in *B. subtilis* provokes a secretion stress response

To investigate whether the secretion stress response is amylase-specific or also provoked by the secretion of other proteins, the induction of a secretion stress response by high-level expression of the secreted *B. subtilis* lipase A (LipA) was investigated. For this purpose, the plasmid pLip2031 directing the overproduction of the LipA protein was introduced into *B. subtilis* 168 *htrB-lacZ*. The transformed strain, when grown in Luria-Bertani medium, showed a growth pattern that was comparable to that of the parental strain 168 (Fig. 3A; ◇ and -). Interestingly, the overproduction of LipA had no significant effect on *htrB-lacZ* transcription as determined by β -galactosidase activity measurements (Fig. 3B; ◇ and -). Furthermore, 2D gel electrophoretic analyses of the extracellular proteome under conditions of LipA overproduction showed no increased concentrations of extracellular HtrA (Jongbloed *et al.*, 2002; unpublished observations). These observations suggested that LipA overproduction may not

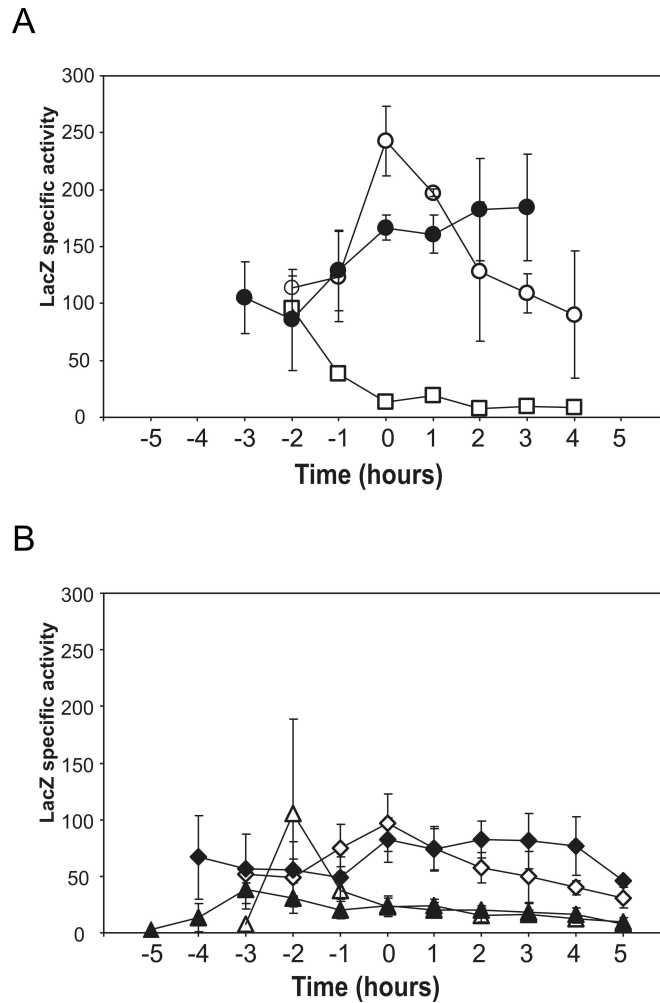


Figure 2. The absence of extracellular proteases has no obvious effect on the *B. subtilis* secretion stress response

To study the effects of the absence of eight proteases from *B. subtilis* WB800 on the secretion stress response, transcriptional *htrA-lacZ* (A) or *htrB-lacZ* (B) fusions were used. Time courses of *lacZ* expression were determined by analysing β -galactosidase activity (indicated in nmol/min/A₆₀₀) in cells grown in Luria–Bertani medium at 37 °C.

Samples were withdrawn at the times indicated; zero time is defined as the transition point between exponential and post-exponential growth. The strains used for the analyses in (A) were: *B. subtilis* 168 *htrA-lacZ* (○); *B. subtilis* 168 *htrA-lacZ* *cssS* (□); and *B. subtilis* WB800 *htrA-lacZ* (●). The strains used for the analyses in (B) were: *B. subtilis* 168 *htrB-lacZ* (◇); *B. subtilis* 168 *htrB-lacZ* *cssS* (△); *B. subtilis* WB800 *htrB-lacZ* (◆), and *B. subtilis* WB800 *htrB-lacZ* *cssS* (▲).

trigger a secretion stress response in *B. subtilis* 168. Notably, however, experiments aimed at determining the production level of mature LipA in the growth medium of *B. subtilis* 168 on overnight growth in Luria-Bertani medium revealed that the LipA concentration was about 0.5 mg/l or even lower (data not shown). This may imply that the LipA production at these levels is simply too low to provoke a detectable secretion stress response.

To verify this idea, plasmid pLip2031 was introduced into the *B. subtilis* WB800 *htrB-lacZ* strain, as earlier studies have demonstrated that LipA is produced at 2.5- to 3-fold higher levels by *B. subtilis* WB800 than the parental strain 168 (Westers *et al.*, 2005). Next, the transcription of *htrB-lacZ* was analysed by determining β -galactosidase activity as a function of time. When the different strains were grown in Luria-Bertani medium, they showed comparable growth rates, but entry into the exponential phase of *B. subtilis* WB800 *htrB-lacZ* pLip2031 cells was delayed (Fig. 3A; \blacklozenge). As shown in Fig. 3C, WB800 cells overproducing LipA (\blacklozenge) did not transcribe *htrB-lacZ* at significantly raised levels compared with the WB800 control strain producing wild-type concentrations of LipA (X), although the data suggest that *htrB-lacZ* expression levels in the cells overproducing LipA were slightly increased.

To verify whether the production of LipA at even higher levels would result in a significant secretion stress response, *B. subtilis* 168 *htrB-lacZ* pLip2031 and WB800 *htrB-lacZ* pLip2031 cells were grown in MXR medium, which has been shown to be an optimal medium for LipA production (Lesuisse *et al.*, 1993). Notably, when cells of *B. subtilis* 168 or WB800 are cultured in this medium (Fig. 3D), they grow at a much slower rate and display an extended exponential growth phase compared with growth in Luria-Bertani medium (Fig. 3A). As shown by β -galactosidase activity determinations, only a mild secretion stress response was induced in LipA-overproducing cells of *B. subtilis* 168 *htrB-lacZ* grown in MXR medium (Fig. 3E; \diamond). In contrast, LipA-overproducing cells of *B. subtilis* WB800 *htrB-lacZ* (Fig. 3F; \blacklozenge and \blacksquare) displayed a clear secretion stress response when grown in MXR medium. Note that in Fig. 3F the curve with closed diamonds represents the average of three datasets, whereas the curve with closed rectangles represents one single outlier dataset which resulted from the variation in LipA production levels that can occur between different 'biological repeats' (Westers *et al.*, 2005). Interestingly, the basal level of *htrB-lacZ* expression in *B. subtilis* 168 or WB800 grown in MXR medium was higher than when these strains were grown in Luria-Bertani medium (Fig. 3; compare panels B and E, or panels C and F). Importantly, as shown with a WB800 *htrB-lacZ* *cssS* mutant strain, the increase in *htrB-lacZ* expression in LipA-overproducing WB800 cells grown on MXR medium was *CssS*-dependent

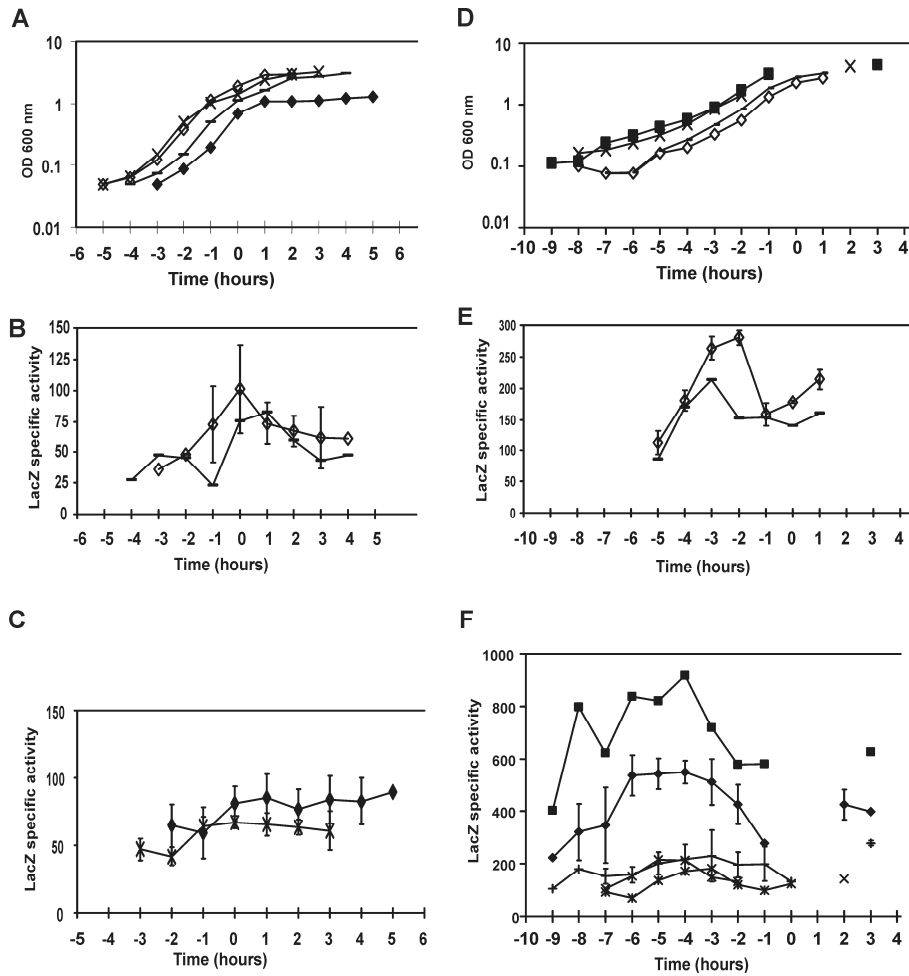


Figure 3. The LipA-induced secretion stress response in *B. subtilis*

A transcriptional *htrB-lacZ* gene fusion was used to determine the time courses of *htrB* expression in *B. subtilis* 168 and WB800 derivatives producing the endogenous LipA directed by the plasmid pLip2031. Cells were grown at 37 °C in LB medium (A-C) or in the lipase overexpression medium MXR (D-F). Growth curves in LB medium (A) or MXR medium (D) were determined by A_{600 nm} readings. Time courses of *htrB-lacZ* expression were determined by analysing β -galactosidase activity (indicated in nmol/min/A₆₀₀) in cells grown in LB medium (B and C) or in MXR medium (E and F). Samples were withdrawn at the times indicated; zero time is defined as the transition point between exponential and post-exponential growth. The strains used were *B. subtilis* 168 *htrB-lacZ* (-), 168 *htrB-lacZ* pLip2031 (◇), WB800 *htrB-lacZ* (X), WB800 *htrB-lacZ* pLip2031 (◆ and ■), WB800 *htrB-lacZ* *cssS* (*), and WB800 *htrB-lacZ* *cssS* pLip2031 (+). Please note that the y-axis (LacZ specific activity) scales are different in panels B, C, E, and F.

(Fig. 3F; +), showing that LipA production provokes a genuine secretion stress response under these conditions. Moreover, the measurement of LipA activity in growth medium samples withdrawn at $t = 3$ from the four parallel MXR cultures of LipA-overproducing WB800 *htrB-lacZ* cells revealed that the outlier culture with the highest *htrB-lacZ* expression level produced about 1.5-fold more LipA than the three other cultures. This indicates that the intensity of the LipA-induced secretion stress response parallels the LipA production levels. Based on SDS-PAGE, using a calibration curve of purified LipA, we estimated the average concentration of LipA in the growth medium of overnight cultures of *B. subtilis* WB800 *htrB-lacZ* pLip2031 grown in MXR medium to be ~11 mg/l, and the LipA production by *B. subtilis* 168 *htrB-lacZ* under these conditions was about twofold lower (data not shown).

For comparison, the level of AmyQ production as directed by plasmid pKTH10L in *B. subtilis* WB800 was estimated to be about 30 mg/l when cells were grown overnight in Luria–Bertani broth (Fig. 4). This level of AmyQ production resulted in a secretion stress response that was comparable to the LipA-induced stress response of WB800 cells grown in MXR medium.

Human interleukin-3 (hIL-3) production provokes a mild secretion stress response in *B. subtilis* 168

To study further the specificity of the *B. subtilis* secretion stress response, the heterologous protein (hIL-3) was produced in *B. subtilis*. The pP43LatIL3 expression system was used for this purpose, because it directs secretion of hIL-3 to about 11 mg/l by the protease-deficient *B. subtilis* strain WB800 grown in Luria–Bertani broth (Fig. 4) (Chapter 3, Westers *et al.*, 2006b). In contrast, the production of hIL-3 by the parental strain 168 is about 10-fold lower because of proteolysis of the secreted hIL-3 (Chapter 3, Westers *et al.*, 2006b). To monitor a possible secretion stress response on hIL-3 production, the plasmid pP43LatIL3 was introduced into the *B. subtilis* strains 168 *htrB-lacZ* and WB800 *htrB-lacZ*, respectively. Next, the expression of the *htrB-lacZ* gene fusions in these strains was analysed by β -galactosidase activity determinations at hourly intervals during growth in Luria–Bertani broth. Interestingly, the *htrB-lacZ* transcription in the 168 strain was slightly increased on production of hIL-3 (Fig. 5B; \triangle), even though the actual yield of hIL-3 in this strain is very low. The expression of *htrB-lacZ* was more clearly increased when hIL-3 was produced in the WB800 strain (Fig. 5C; \blacktriangle), which supports the view that a protein of eukaryotic origin can also provoke a secretion stress response in *B. subtilis*. These increased levels of *htrB* transcription were CssS-dependent (data not shown).

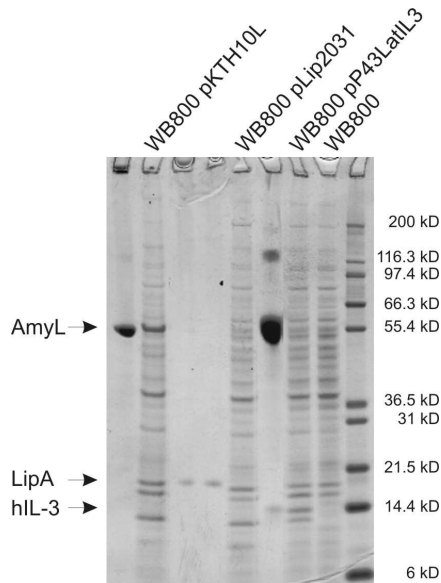


Figure 4. Production levels of AmyQ, LipA and hIL-3 in *B. subtilis* WB800

To visualise the production levels of AmyQ, LipA and hIL-3 in *B. subtilis* WB800 cells grown at 37 °C in Luria-Bertani medium, SDS-PAGE was performed with undiluted growth medium fractions of overnight cultures. For this purpose, *B. subtilis* WB800 was transformed with pKTH10L, pLip2031 or pP43LatIL3, respectively. The amounts of AmyQ, LipA, or hIL-3 present in the medium fractions were determined by densitometric analyses of stained gels. As a reference different amounts of purified AmyL (400 ng), LipA (25 ng and 50 ng) and hIL-3 (15 ng) were loaded on the gel. Note that the commercial reference sample for hIL-3 (Sigma-Aldrich) contains large amounts of BSA for the stabilisation of hIL-3, which forms a band at ~60 kDa.

To verify whether the production of hIL-3 in *B. subtilis* cells at even higher levels would increase the intensity of the secretion stress response, cells of *B. subtilis* 168 *htrB-lacZ* pP43LatIL3 or WB800 *htrB-lacZ* pP43LatIL3 were grown in MSR medium, which has been shown to be optimal for hIL-3 production (Chapter 3, Westers *et al.*, 2006b). The results presented in Fig. 5 (panel A and D) show that, compared with growth in Luria-Bertani medium, significantly higher A_{600} values were reached when the strains were grown in MSR medium. Importantly, the concentrations of hIL-3 produced on overnight growth of *B. subtilis* 168 *htrB-lacZ* pP43LatIL3 and WB800 *htrB-lacZ* pP43LatIL3 in MSR were estimated to amount to ~2 mg/l and ~27 mg/l, respectively (data not shown). As the production of hIL-3 by the 168 cells grown in MSR medium remained relatively low, only the *htrB-lacZ* expression in hIL-3-producing WB800 cells was measured. The results show that, compared with WB800 *htrB-lacZ* cells grown in Luria-Bertani medium (Fig. 5C; X), the basal level of *htrB-lacZ* expression was increased when these cells were grown in MSR medium (Fig. 5E; X). Importantly, WB800 *htrB-lacZ* cells producing hIL-3 displayed increased levels of *htrB* expression (Fig. 5E; ▲), showing that the production of hIL-3 can elicit a secretion stress response in *B. subtilis*.

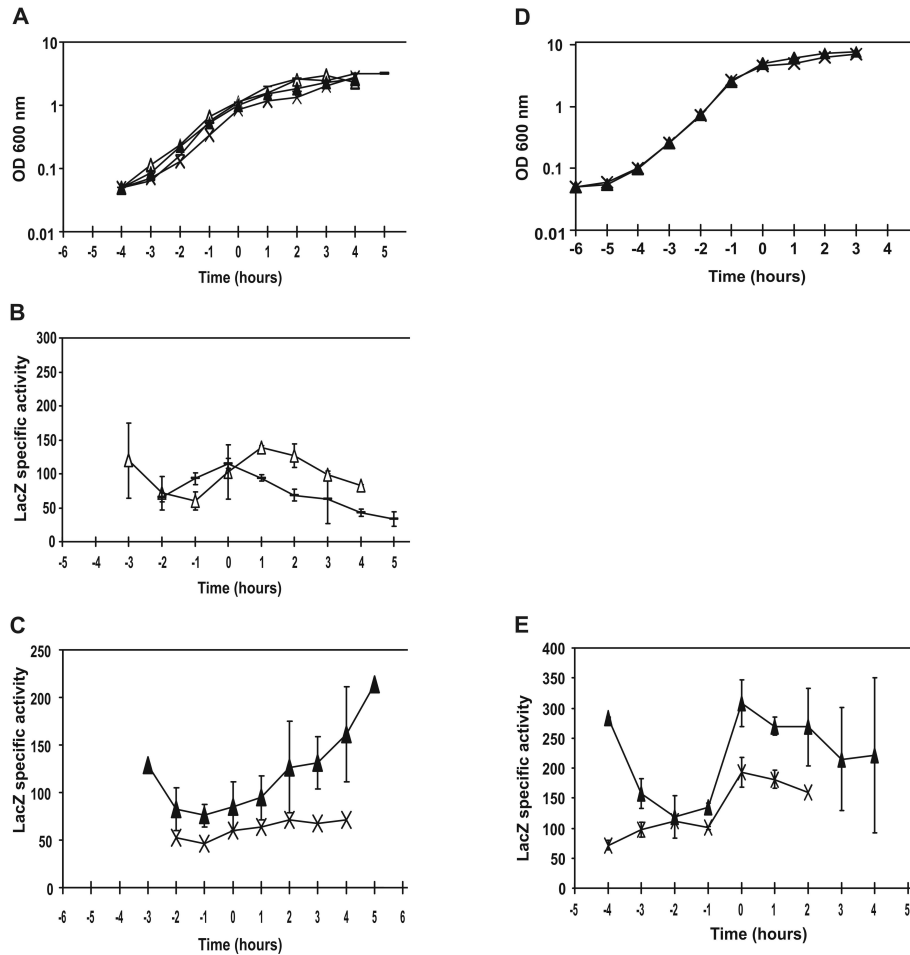


Figure 5. Human Interleukin-3-induced secretion stress response in *B. subtilis*

A transcriptional *htrB-lacZ* gene fusion was used to determine the time courses of *htrB* expression in *B. subtilis* 168 and WB800 derivatives producing hIL-3 directed by the plasmid pP43LatIL3. Cells were grown at 37 °C in LB medium (A-C) or in the hIL-3 overexpression medium MSR (D-E). Growth curves in LB medium (A) or MSR medium (D) were determined by $A_{600\text{ nm}}$ readings. Time courses of *htrB-lacZ* expression were determined by analysing β -galactosidase activity (indicated in nmol/min/ OD_{600}) in cells grown in LB medium (B and C) or in MSR medium (E). Samples were withdrawn at the times indicated; zero time is defined as the transition point between exponential and post-exponential growth. The strains used were *B. subtilis* 168 *htrB-lacZ* (-), 168 *htrB-lacZ* pP43LatIL3 (Δ), WB800 *htrB-lacZ* (X), and WB800 *htrB-lacZ* pP43LatIL3 (\blacktriangle). Please note that the y-axis (LacZ specific activity) scales are different in panels B, C, and E.

DISCUSSION

These studies, which build on previous work concerning the α -amylase-induced CssRS-dependent protein secretion stress response in *B. subtilis*, were aimed at answering two important questions, (a) is α -amylase translocation across the membrane required to trigger this stress response? (b) Is the CssRS-dependent response a general protein secretion stress response? The present observations show that α -amylase translocation is required to trigger a CssRS-dependent stress response, and that production of proteins other than α -amylases can also provoke this protein secretion stress response in *B. subtilis*. Therefore, we conclude that the CssRS-dependent response can be regarded as a general secretion stress response.

The conclusion that non-translocated AmyQ does not provoke a protein secretion stress response is based on the use of the AmyQ-Ala precursor, which contains an artificial alanine-rich signal peptide. This artificial signal peptide is functional in AmyQ translocation in *E. coli*, but not functional in *B. subtilis* (Zanen *et al.*, 2005). The observation that non-translocated AmyQ-Ala does not trigger a secretion stress response is consistent with computer-assisted predictions that indicate that the CssS sensor domain is located at the extracytoplasmic side of the membrane. This suggests that an extracytoplasmic stimulus is sensed by CssS (Hyyryläinen *et al.*, 2001). Interestingly, *B. subtilis* cells overexpressing AmyQ-Leu, which contains a leucine-rich signal peptide, displayed a less intense secretion stress response than cells overproducing the wild-type AmyQ. This observation can be attributed to the fact that AmyQ-Leu is produced at lower concentrations than wild-type AmyQ, as it was previously shown that the intensity of the secretion stress response correlates with the AmyQ production level (Westers *et al.*, 2004a). In this respect, it is noteworthy that no secretion stress response was triggered by AmyQ-Ala, despite the fact that this protein accumulated in the cells at significantly higher levels than AmyQ-Leu, or the wild-type AmyQ. This underscores our view that non-translocated AmyQ neither directly nor indirectly represents a stimulus of the CssS sensor protein. In view of the predicted membrane association of CssS and the demonstrated membrane association of HtrA and HtrB, it seems likely that translocated forms of α -amylase that have not yet been released into the growth medium represent the most effective stimuli for the α -amylase-induced CssRS-dependent secretion stress response. Probably, these cell-associated forms of α -amylase are not (yet) folded, or are misfolded, because mutations in *prsA* that interfere with effective folding of AmyQ result in a more intense

secretion stress response (Hyyryläinen *et al.*, 2001). Nevertheless, we cannot at present exclude the possibility that correctly folded AmyQ can trigger a secretion stress response before its release into the growth medium.

The intensity of the secretion stress response induced on LipA overproduction was found to correlate with LipA production levels, similar to what was previously shown for AmyQ (Westers *et al.*, 2004a). This became particularly evident on cultivation of LipA-overproducing WB800 cells in the MXR medium, a growth medium optimised for LipA production. This suggests that, on increased LipA production, the stimulus that triggers the CssRS-dependent response is also enhanced. Interestingly, a different effect was observed on hIL-3 production. Even though hIL-3 is barely detectable on a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel when produced in *B. subtilis* 168, the expression of the *hIL-3* gene from plasmid pP43LatIL3 is sufficient to provoke a mild secretion stress response. This response is increased, but not dramatically, on 10-fold increased production of hIL-3 in the WB800 strain. These findings suggest that the stimulus that triggers a secretion stress response on hIL-3 production is not proportionally increased with the improved hIL-3 production because of the absence of eight extracellular proteases from the WB800 strain. A possible explanation for this phenomenon is that the secretion stress response is triggered by slowly folding or malformed hIL-3, while both the unfolded and folded hIL-3 are substrates for the extracellular proteases. Thus, removal of the extracellular proteases would impact only mildly on the hIL-3-derived secretion stress stimulus, but heavily on the final yield of hIL-3. In this respect, it is noteworthy that hIL-3 contains one intramolecular disulphide bond. Recent studies have shown that this disulphide bond is properly formed in the hIL-3 produced by *B. subtilis* (Chapter 3, Westers *et al.*, 2006b). It is currently not known, however, whether this important folding step sets a limit to the hIL-3 production level.

In conclusion, these observations show that the CssRS-dependent stress response is a general protein secretion stress response that can be triggered by both homologous (*e.g.* LipA) and heterologous (*e.g.* AmyQ and hIL-3) proteins. The intensity of this response can, to some extent, be correlated with the production level of the secreted protein. Nevertheless, other parameters, such as the dependence of secretory proteins on certain extracytoplasmic folding catalysts or their susceptibility to extracellular proteases, probably determine to what extent the production levels of these secretory proteins and the intensity of the secretion stress response can be correlated. Clearly, the extracellular amount of a particular secretory protein may be much lower than the amount that is actually synthesised because of degradation by cell-associated proteases on membrane translocation. Moreover, the high-level production and

secretion of one particular protein may impact on the rates of translocation and the quality of folding of certain secretory proteins of the host cell. Therefore, future research should address the question whether secretion stress is mainly due to the accumulation of folded or misfolded secretory proteins at the membrane-cell wall interface, or to the rates of translocation and subsequent folding of the translocated proteins. These are important considerations in attempts to apply the secretion stress response as an indicator for the optimised production and quality of biotechnologically relevant secretory proteins in *Bacillus* species.

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Nog een hoofdstuk over stress dus. Nog even doorbijten!

Over stress gesproken: iedereen die ooit een verbouwing heeft meegemaakt, weet dat, net als bij promotietrajecten, een verbouwing zelden volgens de voorgenomen planning verloopt. De laatste loodjes wegen het zwaarst, maar als het dan ook achter de rug is... heb je ook wat!

Op de foto's staat de volledig vernieuwde dansschool in Delfzijl. Nog niet klaar, zoals gezegd: de laatste loodjes!